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C5a/C5aR1 axis promotes the progression of renal tubulointerstitial fibrosis in a mouse model of renal ischemia/reperfusion injury

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Abstract

C5a is a potent proinflammatory agonist that mediates renal ischaemia reperfusion (IR) injury, but the potential for modulating chronic post ischaemic fibrosis and use of therapeutic antagonist is undefined. Here we show that C5a receptor 1 (C5aR1) signalling is essential to the development of post-ischaemic fibrosis and is a valid target for therapeutic blockade with soluble receptor antagonist. In the present study, we show that C5aR1 is required for the development of renal tubulointerstitial fibrosis in a murine model of renal IR injury. Deficiency of C5aR1 protected mice from the development of the fibrosis. The protection was associated with attenuated deposition of extracellular matrix components (fibronectin, collagen I), reduced cellular infiltrates (CD45, F4/80) and gene expression of proinflammatory and profibrogenic mediators in the kidney. In an *in vitro* model of hypoxia/reoxygenation, C5a stimulation caused renal fibroblast proliferation and activation, and upregulated gene expression of IL-1β, IL-6, TGF-β in renal tubular epithelial cells and monocytes/macrophages. Administration of C5aR1 antagonist (PMX53) significantly reduced renal injury and tubulointerstitial fibrosis. Our results demonstrate a pathogenic role for C5aR1 in progression of tubulointerstitial fibrosis following renal IR injury and provide evidence supporting that C5aR1 mediated-local inflammatory responses to hypoxic renal injury contribute to tubulointerstitial fibrosis through several cellular pathways, namely, promoting tubule injury, interstitial fibroblast proliferation and epithelial-to-mesenchymal transition of renal tubular epithelial cells. Our results also suggest the C5a-C5aR1 interaction is a therapeutic target for chronic post ischaemic fibrosis.

Introduction

Progressive tubulointerstitial fibrosis is the final common pathway for many chronic kidney diseases (CKD) leading to end-stage renal failure. Acute kidney injury (induced for example by such as ischemic insult, systemic inflammation and sepsis) is one of the main causes of CKD, among other causes (e.g. diabetes and hypertension, infections, glomerulonephritis, renal vasculitis)[1]. Renal ischemia reperfusion (IR) injury is an inevitable consequence of kidney transplantation and contributes to the progression of graft dysfunction and renal fibrosis [2]. As there are currently no specific treatments for tubulointerstitial fibrosis, identifying key mediators of renal fibrosis will be beneficial to the development of effective strategies that prevent or limit the fibrosis in chronic inflammatory states.

Tubulointerstitial fibrosis is characterized by progressive replacement of the normal architecture of tubules is progressively replaced with extracellular matrix (ECM) including collagen I (COL I) and fibronectin (FN). Excessive deposition of those proteins leads to structural and functional change of the kidney parenchyma and eventually renal dysfunction [3]. The major cell type of producing ECM is

the myofibroblast which produces a large amount of COL I and FN [4, 5]. Myofibroblasts mainly differentiate from renal interstitial fibroblasts, other types of cells in the kidney such as bone marrow-derived fibrocystic and stromal mesenchymal cells, and renal tubular epithelial cells have been reported to be able to transform into myofibroblasts [6]. The pathogenesis of tubulointerstitial fibrosis involves many elements (i.e. cytokines, growth factors, leukocytes, activation of fibroblasts, epithelial-to-mesenchymal transition [EMT]) are thought to contribute to the development of progressive tubulointerstitial fibrosis [7, 8]. However, recent research has highlighted the role of the interstitial fibroblast and its activation and differentiation into myofibroblast in promoting renal tubulointerstitial fibrosis, excessive or persistent inflammation being an important driver of fibroblast activation[4, 5, 9].

Complement activation occurs rapidly in response to hypoxic renal injury and other types of noxious agents including physical and chemical stress [10-12]. C5a, generated during complement activation is a potent inflammatory mediator which attracts leukocytes to the site of the injury**.** Through binding to its specific receptor complement C5a receptor 1 (C5aR1), which is expressed on wide variety of cells including myeloid and non-myeloid cells, C5a triggers the release of inflammatory cytokines and other substances inducing local inflammation. Thus, C5a/C5aR1 interactions play important roles in regulating/coordinating many inflammatory and immunological processes that contribute to the pathogenesis of a wide range of inflammatory and immunological diseases [13-15].

Previous studies by us and others have shown that C5a/C5aR1 interactions mediate the pathogenesis of renal IR injury and allograft rejection [13, 16-18]. However, little is known about the role of C5aR1 in progression of tubulointerstitial fibrosis following renal IR injury and the underlying mechanisms. Given early inflammatory responses to hypoxic renal injury not only cause tissue damage, but also contribute to chronic inflammation and chronic graft dysfunction, we hypothesised that C5a/C5aR1 interactions could play a role in progression of tubulointerstitial fibrosis following renal IR injury and could provide a tractable therapeutic target.

In the present study, we employed a murine model of renal IR injury in *C5aR1*$^{-/-}$ mice to determine the role of C5aR1 in the development of renal tubulointerstitial fibrosis. We also performed a series of *ex vivo* analyses and *in vitro* experiments (using primary cultures of murine renal tubular epithelial cells [RTEC], renal fibroblasts and peritoneal exudate leukocytes) to explore the mechanisms by which C5a/C5aR1 interactions influence the development of tubulointerstitial fibrosis. Our results demonstrate a pathogenic role for C5aR1 in the development of tubulointerstitial fibrosis and suggest that C5a/C5aR1 interactions promote progression of the fibrosis through mediating local inflammatory responses and directly stimulating renal fibroblasts.

Results

C5aR1-/- mice have reduced renal tubulointerstitial fibrosis following renal IR insult

We first examined the time-kinetics of the development of the fibrosis in WT mice following renal IR insult which was induced by clamping renal arteries and veins bilaterally for 35 min. Renal tubulointerstitial fibrosis was assessed at 5, 10 and 20 days after reperfusion by Sirius red (SR) and Masson's trichrome (MT) staining. The degree of tubulointerstitial SR- or MT-positively stained area was increased over this period, with a sharp increase at day 10 (Figure 1A), indicating the day 10 after reperfusion is a suitable time point for analysing tubulointerstitial fibrosis in this model. Next, we assessed the fibrosis in WT and *C5aR1-/-* mice at day 10 after reperfusion. SR staining showed that the positively stained area was significantly reduced in *C5aR1*-/- kidneys, compared to WT kidneys, indicating a reduced tubulointerstitial deposition of extracellular matrix (ECM) in *C5aR1*-/- kidneys (Figure 1B, C). Immunohistochemistry further showed that *C5aR1*-/- kidneys had a reduced deposition of collagen I (COL I) and fibronectin (FN), compared to WT kidneys (Figure 1D, E). Consistent with the immunohistochemistry results, RT-qPCR showed that intrarenal gene expression levels of COL I and FN were significantly lower in *C5aR1*-/- mice than in WT mice (Figure 1F). We also monitored the changes of renal function at 5, 10 and 20 days after reperfusion by measuring BUN levels. Renal function at days 5 and 10 was better preserved in the deficient mice compared to WT, but the difference was less apparent by day 20 after the acute IR injury (Figure 1G). Thus, C5aR1 deficient mice had reduced renal tubulointerstitial fibrosis and better preserved renal function following renal IR insult, suggesting pathogenic roles for C5aR1 in the development of renal tubulointerstitial fibrosis.

C5aR1 deficiency has an impact on leukocyte infiltration and macrophage phenotype in the kidney following renal IR insult

Renal interstitial leukocyte infiltration is a hallmark of renal inflammation in renal IR injury. We therefore assessed the impact of C5aR1 on leukocyte infiltration and macrophage phenotype in the kidneys through to the chronic phase after renal IR insult. Our previous study (of the acute injury) has shown that the basal levels of renal leukocytes in normal WT and *C5aR1-/-* mice were comparable[16]. Here, flow cytometry analysis showed that *C5aR1*-/- mice had significantly less CD45+ (total leukocytes) and F4/80^{+} (macrophages) cells at 5, 10 and 20 days after reperfusion, compared to WT mice (Figure 2A-D). Macrophage phenotype plays an important role in the processes of tissue repair and fibrosis following acute kidney injury [9]. Previous studies have suggested that CD11b^{+}F4/80^{low} cells are M1 like macrophages, while CD11b^{+}F4/80^{high} are M2 like macrophages [19, 20]. We therefore further analysed macrophage phenotype in the kidneys of WT and *C5aR1*-/- mice at day 10 after reperfusion. Flow cytometry analysis showed that *C5aR1*-/- kidneys had significantly more F4/80^{high} cells and less F4/80^{low} cells as well as higher ratio of F4/80^{high} to F4/80^{low}, in the CD11b^{+} compartment, compared to WT kidneys (Figure 2E-F). These data

demonstrate that C5aR1 deficiency not only reduced leukocyte infiltration but also favoured the differentiation of M2-like phenotype versus M1-like phenotype in macrophages.

C5aR1-/- mice have reduced intrarenal gene expression of proinflammatory and profibrotic mediators following renal IR insult

C5aR1 signalling is responsible for inflammatory responses in the acute phase of renal IR injury [13, 16]. However, its involvement in renal tissue inflammation and fibrogenesis in the late phase of the injury is less certain. We therefore assessed the impact of C5aR1 on renal tissue inflammation and fibrogenesis by performing RT-PCR for the molecules relevant to renal fibrosis in kidneys of WT and *C5aR1-/-* mice at 5 and 10 days after reperfusion. Intrarenal mRNA levels of pro-inflammatory cytokines (IL-1β, TNF-α), chemokine (MCP-1) and chemokine receptor (CCR2) were significantly lower in *C5aR1-/-* mice compared to WT mice (Figure 3A). Conversely, intrarenal mRNA levels of anti-inflammatory cytokine (IL-10) were higher in *C5aR1-/-* mice (Figure 3B). Intrarenal mRNA levels of profibrogenic mediators (TGF-b, PDGF) were also lower in *C5aR1-/-* mice than in WT mice, which correlates well with observed changes in pro-inflammatory mediators (Figure 3C). These results indicate that C5aR1 deficiency resulted in a reduction of intrarenal expression of pro-inflammatory and profibrogenic mediators in the late phase of renal IR injury, suggesting a role for C5aR1 in tissue inflammation and fibrogenesis.

C5aR1-/- mice exhibit an attenuated epithelial-to-mesenchymal transition of renal tubular epithelial cells following renal IR insult

Epithelial-to-mesenchymal transition (EMT) is recognised as an integral part of tissue fibrogenesis after injury [6], which is often triggered by and associated with chronic/persistent local inflammation [7]. Given that C5a/C5aR1 interactions are an important driver of inflammation, and *C5aR1-/-* mice had decreased renal tissue inflammation in the late phase of renal IR injury as observed above, we hypothesised that C5aR1 deficiency attenuates EMT of tubular epithelial cells following renal IR insult. We performed immunohistochemistry for E-cadherin (E-Cad, an epithelial marker) and fibroblast-specific protein 1 (FSP-1, a mesenchymal marker) in the injured kidneys of WT and *C5aR1-/-* mice (10 days after reperfusion). C5aR1-/- kidneys exhibited higher E-Cad and lower FSP-1 expression (Figure 4A, 4B) as well as significantly lower ratio of FSP-1 to E-Cad expression compared to WT kidneys (Figure 4C), suggesting a reduction of EMT of RTEC in *C5aR1-/-* mice following renal IR insult.

C5a up-regulates pro-inflammatory cytokine and pro-fibrotic factor production by hypoxia stressed-RTEC and - MO/MΦ in vitro

RTEC and monocytes/macrophages (MO/MΦ) are an important source of proinflammatory and profibrogenic mediators [21, 22]. Our previous study has shown that C5a stimulation increases the murine IL-8 homologues (KC) and TNF-α production by macrophages and RTEC under hypoxia-

reoxygenation conditions[16]. Here, we assessed the effects of C5a on hypoxia/reoxygenation-induced production of several additional proinflammatory and profibrogenic mediators (i.e. IL-1β, IL-6 and TGF-β) by these two types of cells. RT-qPCR showed that mRNA levels of these mediators were significantly increased in RTEC and MO/MΦ following C5a stimulation (Figure 5). These results demonstrate that C5a has direct effects on RTEC and MO/MΦ to up-regulate proinflammatory cytokine and profibrogenic mediator production under hypoxia-reoxygenation condition.

C5a stimulates renal fibroblast proliferation and ECM production

The activation of interstitial fibroblasts to become myofibroblasts is an essential step in the development of renal fibrosis. Expression of C5aR1 has been reported in fibroblast of lung and periodontium [23, 24]. We hypothesised that renal fibroblasts may also express C5aR1 and C5a/C5aR1 interactions may have effects on cell proliferation and activation. To test this, we first confirmed the expression of C5aR1 in primary renal fibroblasts by immunocytochemistry and RT-PCR. Immunochemical staining showed that renal fibroblasts exhibited a fibroblast-like morphology and positive staining for the mesenchymal marker vimentin, and C5aR1 was clearly detected in these cells (Figure 6A). Expression of C5aR1 in renal fibroblasts was further confirmed by RT-PCR. Results showed that C5aR1 was detected under normal conditions and the expression was increased in response to hypoxic stress (Figure 6B, 6C). Next, we assessed the effect of C5a on renal fibroblast proliferation and ECM production. Proliferation assay showed that addition of C5a increased the proliferation of renal fibroblasts (Figure 6D). RT-qPCR showed that addition of C5a upregulated gene expression of *COL I*, *FN* and *αSMA* in renal fibroblasts (Figure 6E). These data suggest that C5a/C5aR1 interaction has a stimulatory effect on renal fibroblast proliferation and activation.

Blocking C5aR1 with antagonist (PMX53) protects mice from developing renal tubulointerstitial fibrosis following renal IR insult

Having shown that C5a/C5aR1 interactions are required for the development of tubulointerstitial fibrosis by using *C5aR1*-/- mice, next we explored the therapeutic potential of targeting C5aR1 in tubulointerstitial fibrosis. We employed PMX53 (a recognized C5aR1 antagonist) in our renal IR injury mouse model. PMX53 treatment (i.p. daily) started on the day before the induction of injury significantly reduced tubulointerstitial deposition of ECM (as measured by SR staining and immunohistochemistry for COL I and FN) and tubule damage (as measured by LTL staining) (10 days after reperfusion) (Figure 7A-D). A concomitant improvement in renal function was observed in PMX53 treated mice compared with control-treated group (Figure 7E). We also examined if delayed administration of PMX53 would reduce renal fibrosis following renal IR insult and performed an additional set of experiments including three groups of mice: i) control treatment, ii) PMX53 treatment starting at day 0 (d0-d9), iii) PMX53 treatment starting at day 3 (d3-d9). Renal fibrosis and function were assessed 10 days after reperfusion. Intrarenal gene expression of COL I and FN and

tubulointerstitial deposition of ECM were reduced in both PMX53 treatment groups compared to control-treated group, this was accompanied by improvement in renal function in both PMX53 treatment groups. PMX53 treatment starting at day 0 appears more effective than the treatment starting at day 3 (Figure 7F-H). Together, these results demonstrate that blocking C5aR1 with antagonist confers protection against the development of renal fibrosis following renal IR insult. The results were consistent with the observations in *C5aR1-/-* mice, further confirming a role of C5aR1 in the development of tubulointerstitial fibrosis.

Discussion

Inflammatory responses to hypoxia can cause tissue damage and contribute to tissue fibrosis and chronic renal dysfunction. Identification of key mediators of inflammatory responses driving fibrotic process following renal IR injury may lead to improvement of renal function through specific intervention. Although there is compelling evidence on the pathogenic roles of C5a/C5aR1 signalling in acute renal IR injury, graft dysfunction and allograft rejection [16-18], the impact of the signalling on progression of renal fibrosis following renal IR injury has been unclear. The present study provides evidence that C5a/C5aR1 signalling is required for the development of tubulointerstitial fibrosis following renal IR insult, thus, supporting a pathogenic role for C5a/C5aR1 signalling in renal fibrosis. This also suggests a potential link between C5aR1 dependent renal IR injury and chronic transplant dysfunction, where a role for C5aR1 signalling has been implicated in acute post-transplant injury.

Pathogenic roles for C5aR1 in renal fibrosis have been reported in the unilateral ureteral obstruction model and chronic pyelonephritis model in mice by us and others [22, 25, 26]. However, the insults in those models are different from the insult in this study; the kinetics of cellular responses to the insult, the major pathological parameters and the involved mechanisms in those models might be different from that in IR injury model. In the present study, we performed a series of *in vivo* experiments to titrate the kinetics of interstitial collagen deposition following renal IR injury. We found that the d10 after reperfusion is an optimal time point to analyse interstitial collagen deposition by SR, TM and immunohistochemistry for COL I and FN, whereas d5 and d10 are optimal time points to analyse renal cellular infiltrates and intrarenal gene expression of proinflammatory and pro fibrogenic mediators. The results of our *in vivo* experiments, using mice with genetic deletion or through pharmacologic inhibition of C5aR1 and by measuring multiple parameters (i.e. collagen deposition, cellular infiltration and intrarenal gene expression, renal function), clearly show that C5aR1 is required for the development of tubulointerstitial fibrosis following renal IR insult, thus supporting a pathogenic role for C5aR1 in this process.

In the present study, in addition to determining the role of C5R1 in tubulointerstitial fibrosis, we investigated the mechanisms by which C5a/C5R1 signalling contributes to the pathogenesis of tubulointerstitial fibrosis, by focusing specifically on cellular mechanisms. As illustrated in Figure 8, our data suggest that, in response to renal IR insult, C5a/C5aR1 interactions may promote progression of tubulointerstitial fibrosis through acting on inflammatory cells, renal tubular epithelial cells and renal fibroblasts and contributing to the proinflammatory environment in tubulointerstitium and fibroblast proliferation/activation. More specifically, first, our *ex vivo* analysis of injured renal tissues showed that C5aR1 deficiency not only reduced leukocyte infiltration and macrophage accumulation, but also favoured the differentiation of macrophages into a M2-like phenotype, this is associated with a concomitant reduction in intrarenal gene expression of proinflammatory and profibrogenic mediators. These results provide support for the roles of C5aR1 in mediating local inflammation and promoting M1 macrophage in this model, it also suggests a pathogenic role for M1 macrophage in progression of tubulointerstitial fibrosis following renal IR insult, which is in agreement with several published studies that suggest M1 macrophage drives renal fibrosis after AKI, while M2 macrophage is necessary for recovery from AKI [27-29]. On the contrary, some studies also suggested that M2 like macrophages may contribute to renal fibrosis, particularly in the chronic phase of kidney injury [30, 31], thus highlighting the complicated role of different macrophage subsets in renal repair/fibrosis, more studies on M1/M2 subsets and their relation to *in vivo* recovery process from AKI are needed. In addition, our results obtained from *in vitro* hypoxia experiments showed that the production of proinflammatory and profibrogenic mediators in MO/MΦ was significantly upregulated by C5a stimulation. These results suggest that C5a/C5aR1 signalling, as a potent inflammatory mediator, mediates recruitment and activation of inflammatory cells, thus leading to a proinflammatory environment which may not only cause tubule injury and EMT of RTEC, but also stimulate renal fibroblasts. Second, in addition to the effects on inflammatory cells, our results obtained from *in vitro* hypoxia experiments also showed that the production of proinflammatory and profibrogenic mediators in RTEC was upregulated by C5a stimulation. In addition, EMT of RTEC in the kidney following renal IR insult was markedly reduced in *C5aR1-/-* mice. These results suggest that C5a/C5R1 signalling on RTEC can also contribute to a proinflammatory environment responsible for tubule injury, cellular accumulation and EMT. Third, another important finding in our *in vitro* hypoxia experiments is that renal fibroblast proliferation and ECM production were significantly enhanced by C5a stimulation, suggesting that C5a/C5R1 signalling can contribute to tubulointerstitial fibrosis through its stimulatory effects on renal fibroblast. In agreement with this observation, it has been shown that C5a/C5aR1 signalling has direct effects on pulmonary fibroblasts which could contribute to the pathogenesis of bleomycin-induced lung fibrosis[24]. Results from our C5aR1 blocking experiments, which show that delayed administration of PMX53 (3 days after reperfusion) also reduced renal fibrosis, suggest that C5a/C5aR1 interactions may have direct pro-fibrotic effects, independently of the early inflammatory responses they induced.

In conclusion, the present study demonstrates a pathogenic role for C5aR1 in tubulointerstitial fibrosis following renal IR insult and address the underlying mechanisms of C5aR1 promoting the development of tubulointerstitial fibrosis. Furthermore, the findings present in this study, together with the findings of previous studies about the pathogenic role of C5aR1 in chronic graft dysfunction and allograft rejection, suggest a new avenue for therapeutic targeting in chronic renal or graft injury.

Materials and Methods

Mice

Homozygous C5aR1$^{-/-}$ mice were derived by homologous recombination in embryonic stem cells [32] and backcrossed onto to C57BL/6 (H-2^{b}) strain for more than 12 generations. WT C57BL/6 mice were purchased from Harlan Laboratories (UK). Male mice (8-12 weeks) were used in all the experiments. Animal procedures adhered to the Animals (Scientific Procedures) Act of 1986.

Induction of renal IR injury

Renal IR injury was induced as we previously described with some modifications [33]. In brief, mice were anesthetized by isoflurane and a midline abdominal incision was made. The renal arteries and veins were isolated and bilaterally occluded with micro aneurysm clamps for 35 min. After removal of the clamps, 1 ml of warm saline was put in the abdomen and the incision was sutured. Mice were kept up to 20 days. In some experiments, mice were treated with C5aR1 peptide antagonist (PMX53) [34] or control peptide (1 mg/kg), starting at d0 or d3, daily by intraperitoneal (i.p.) injection. Mice were killed 10 days after reperfusion. In some experiments, at day 5, 10 and 20 after reperfusion, blood samples were taken for renal function assessment and kidneys were collected for histopathology, flow cytometry and RT-qPCR.

Assessment of renal function and fibrosis

Renal function was determined by measuring the blood urea nitrogen (BUN) in the serum using Infinity Urea kit. To assess renal fibrosis, kidney paraffin sections (4 μm) were stained for Sirius red and Masson's trichrome and scanned with a Hamamatsu Nanozoomer 2.0 HT (Hamamatsu Photonics, Hamamatsu, Japan) and viewed using NDP.view2 software. Some images were taken in the cortical medullary junction using an Olympus BX51 microscope (Japan). The positively stained red or blue tissue in each image was quantified by Image J (National Institutes of Health, Bethesda, MD, USA) as percentage of positive staining per field (4 fields from 2 sections per kidney were analysed). The quantitative analyses were performed in a blinded fashion by 2 experienced persons. Collagen deposition was further assessed by immunohistochemistry for Collagen I (COL I) and fibronectin (FN).

Immunohistochemistry and Immunocytochemistry

Immunohistochemistry was performed on kidney sections and renal fibroblasts. Prior to the staining, the formalin-fixed, paraffin-embedded kidney sections (5 μm) were deparaffinised and rehydrated, followed by heat-induced antigen retrieval using sodium citrate or Tris EDTA. The OCT-embedded frozen kidney sections (4 μm) were fixed in ice-cold acetone for 5 min, while the primary renal fibroblasts grown on the coverslips were fixed in 4% paraformaldehyde for 15 min and permeablized by 0.5% Triton X-100 for 10 min. All the tissue or cells were blocked with 10% goat or donkey serum for 30 min, followed by an overnight incubation with primary antibodies (for COL I, FN, E-cadherin, FSP-1, C5aR1 or vimentin) at 4℃ and 1-hour incubation with secondary antibodies (TRITC-conjugated goat anti-rabbit IgG Ab, FITC-conjugated donkey anti-rabbit or anti-rat IgG Ab, Alexa Fluor 568 Donkey anti-goat IgG), LTL (detecting L-fucose in the proximal tubules) and DAPI (staining nuclei) at room temperature. The staining of COL I and FN in the cortical medullary junction was imaged via an A1R point scanning confocal microscope (Nikon, Japan). Quantification were carried out on the images at 600x magnification. The positive staining of E-cadherin and FSP-1 was quantified by Image J as fluorescence intensity per field (six fields from 2 sections per kidney at 100x magnification).

Induction of hypoxia-reoxygenation injury in RTEC and fibroblasts *in vitro*

RTEC and renal fibroblasts were cultured under hypoxic conditions (5% CO_2, 1% O_2 and 94% N_2) in a gas chamber for 24 hours to induce hypoxia stress, followed by reoxygenation (21% O_2 and 5% CO_2) for 24, or 48 hours with or without the presence of C5a. In some experiments, total RNA was extracted and RT-qPCR was performed to assess the expression of pro-inflammatory molecules (IL-1β, IL-6, TGF-β) in RTEC and ECM molecule (Col I, FN and αSMA) in renal fibroblasts.

Statistical analysis

Data are shown as mean ± SD or the readout of individual mice. Unpaired Student's *t* test was used to compare the means of two groups. One-way or Two-way ANOVA was used to compare the means of more than two independent groups. All the analyses were performed using Graphpad Prism 7 software. $P < 0.05$ was considered to be significant.

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Supplementary Material

Supplementary Methods, Supplementary References, Supplementary Table for PCR primer sequences and product sizes.

Supplementary information is available at Kidney International's website.

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Figure legends

Figure 1. C5aR1 deficiency reduces renal tubulointerstitial fibrosis following renal IR insult.
Renal IR injury was induced in WT and *C5aR1*-/- mice. Serum samples and kidneys were collected at d5, d10 and d20 after reperfusion. (**A**) Representative images of Sirius red (SR) and Masson's trichrome (MT) staining on normal and injured WT kidneys at varied time points after reperfusion. Scale bar: 100 μm. (**B**) Representative images of SR on injured WT and *C5aR1*-/- kidneys at d10 after reperfusion. Scale bar: 50 μm. (**C**) Quantification of Sirius red-stained areas corresponding to the WT and *C5aR1*-/- mice in (B). Data were analysed by Unpaired two-tailed Student's t test (n=6 mice per group). (**D, E**) ECM protein deposition in kidneys. (**D**) Representative fluorescence microscope images of COL I (red), FN (red), and lotus tetragonolobus lectin (LTL) (a proximal tubular marker) (green), and DAPI (blue) staining on kidneys of WT and *C5aR1*-/- mice at d10 after reperfusion, taken at the cortical-medullary junction. Arrows indicate positive-stained areas. Scale bars: 25 μm. (**E**) Quantification of positively stained areas of COL I and FN corresponding to the WT and *C5aR1-/-* mice in **D**. Data are shown as mean ± SD and were analysed by One-way ANOVA with multiple comparisons test (n=6-8 viewing fields from 3 mice per group, under x600 magnification). (**F**) Relative mRNA levels of *COL I* and *FN* in the WT and *C5aR1*-/- mouse kidneys at day 10 after reperfusion, determined by RT-qPCR. Data were analysed by Unpaired two-tailed Student's t test (n=5 mice per group). (**G**) BUN levels in WT and *C5aR1*-/- mice at d5, d10 and d20 after reperfusion. Data were analysed by Two-way ANOVA with multiple comparisons test (n=4 to 8 mice per group, each time point). **(C, F, G)** Each dot represents an induvial mouse. Dashed line represents the BUN level in normal mice. **, P<0.01; ****; P< 0.0001.

Figure 2. Impact of C5aR1 deficiency on leukocyte infiltration and macrophage phenotype in the kidney following renal IR insult

(A-D) Inflammatory cell infiltration in WT and *C5aR1*-/- kidneys at d5, d10 and d20 after reperfusion, determined by flow cytometry. (**A**) Representative dot plots of CD45+ cells in the WT and *C5aR1*-/- kidneys at d10. (**B**) Quantification of CD45+ cells at the all-time points. (**C**) Representative dot plots of F4/80+ cells in the WT and *C5aR1*-/- kidneys at d5. (**D**) Quantification of F4/80+ cells at the all-time points. (**B, D**) Data were analysed by Two-way ANOVA with multiple comparisons test (n=5 mice per group, each time point). (**E**) Stepwise gating strategy used in flow cytometric analysis of CD11b+F4/80^{hi} and CD11b+F4/80^{lo} cells in kidney tissues. (**F, G**) Quantification of the proportion of F4/80^{hi} and F4/80^{lo} subset in CD11b+ cells and the ratio of these two types of cells in the WT and *C5aR1*-/- kidneys at d10. Data were analysed by One-way ANOVA with multiple comparisons test (n=4 mice per group and representative of 2 independent experiments). (**B, D, F, G**) Each dot represents an induvial mouse. *, P<0.05; ***, P<0.001; ****, P<0.0001.

Figure 3. C5aR1 deficiency is associated with reduced renal tissue inflammation and fibrogenesis following renal IR insult.

Relative mRNA levels of proinflammatory (**A**), IL-10 (**B**) and profibrogenic factors (**C**) in the WT and *C5aR1-/-* mouse kidneys at d5 and d10 after reperfusion, determined by RT-qPCR. Data were analysed by Two-way ANOVA with multiple comparisons test (n=5 mice per group, each time point). Each dot represents an induvial mouse. *, P<0.05; **, P<0.01.

Figure 4. *C5aR1-/-* mice exhibit an attenuated epithelial-to-mesenchymal transition of renal tubular epithelial cells following renal IR insult

Renal IR injury was induced in WT and *C5aR1-/-* mice. The kidneys were collected at d10 after reperfusion. (**A**) Representative fluorescence microscope images of E-cadherin (E-Cad) (green) and FSP-1 (red), and DAPI (blue) staining in kidney sections of WT and C5aR1-/- mice. Scale bar: 100 μm. (**B**) Quantification of E-Cad and FSP-1 expression in renal tubules corresponding to the WT and *C5aR1*$^{-/-}$ mice in **A** by measuring fluorescence intensity using ImageJ, presented as fluorescence intensity units (FIU). Data were analysed by One-way ANOVA with multiple comparisons test (n= 4 mice per group). (**C**) Ratio of FSP-1 to E-Cad expression in the WT and *C5aR1*$^{-/-}$ mice. Data were analysed by Unpaired two-tailed Student's t test (n=4 mice per group). Each dot represents an induvial mouse. *, P<0.05; ****, P<0.0001.

Figure 5. C5a up-regulates pro-inflammatory cytokine and pro-fibrotic factor production by hypoxic stressed-RTECs and MO/MΦ in vitro.

(**A**) Primary renal tubular epithelial cells (RTECs), and (**B**) peritoneal monocytes/macrophages (MO/MΦ) were undergone hypoxia for 24 hours, followed by 24 hours of re-oxygenation in the presence or absence of C5a. Relative mRNA levels of IL-1β, IL-6 and TGF-β were measured by RT-qPCR. Data are shown as mean ± SD and were analysed by One-way ANOVA with multiple comparisons test (n=4 independent experiments). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.

Figure 6. C5a stimulates renal fibroblast proliferation and activation.

(**A**)Fluorescence microscopy images of primary renal fibroblasts from normal WT mice stained for C5aR1 (red), vimentin (green) and nuclear marker DAPI (blue). Scale bar: 10 μm. (**B**) Detection of C5aR1 mRNA in the fibroblasts by RT-PCR. M, DNA ladder. (**C**) Relative mRNA levels of C5R1 in normal and stressed (had undergone hypoxia for 24h) renal fibroblasts, determined by RT-qPCR. Data are shown as mean ± SD and were analysed by Unpaired two-tailed Student's t test (n=3 mice per group). (**D**) Proliferation of fibroblasts (that had undergone hypoxia for 24h and followed by C5a stimulation for further 48h), assessed by fluorescent reader. Data are shown as mean ± SD and were analysed by One-way ANOVA with multiple comparisons test (n=6 readouts from 3 experiments). (**E**) Relative mRNA levels of *COL I*, *FN* and *α-SMA* in renal fibroblasts that had

undergone 24 hours of hypoxia and 24 hours of re-oxygenation, in the absence or presence of C5a, determined by RT-qPCR. Data are shown as mean ± SD and were analysed by One-way ANOVA with multiple comparisons test (n=3 independent experiments). *, P<0.05; **, P<0.01.

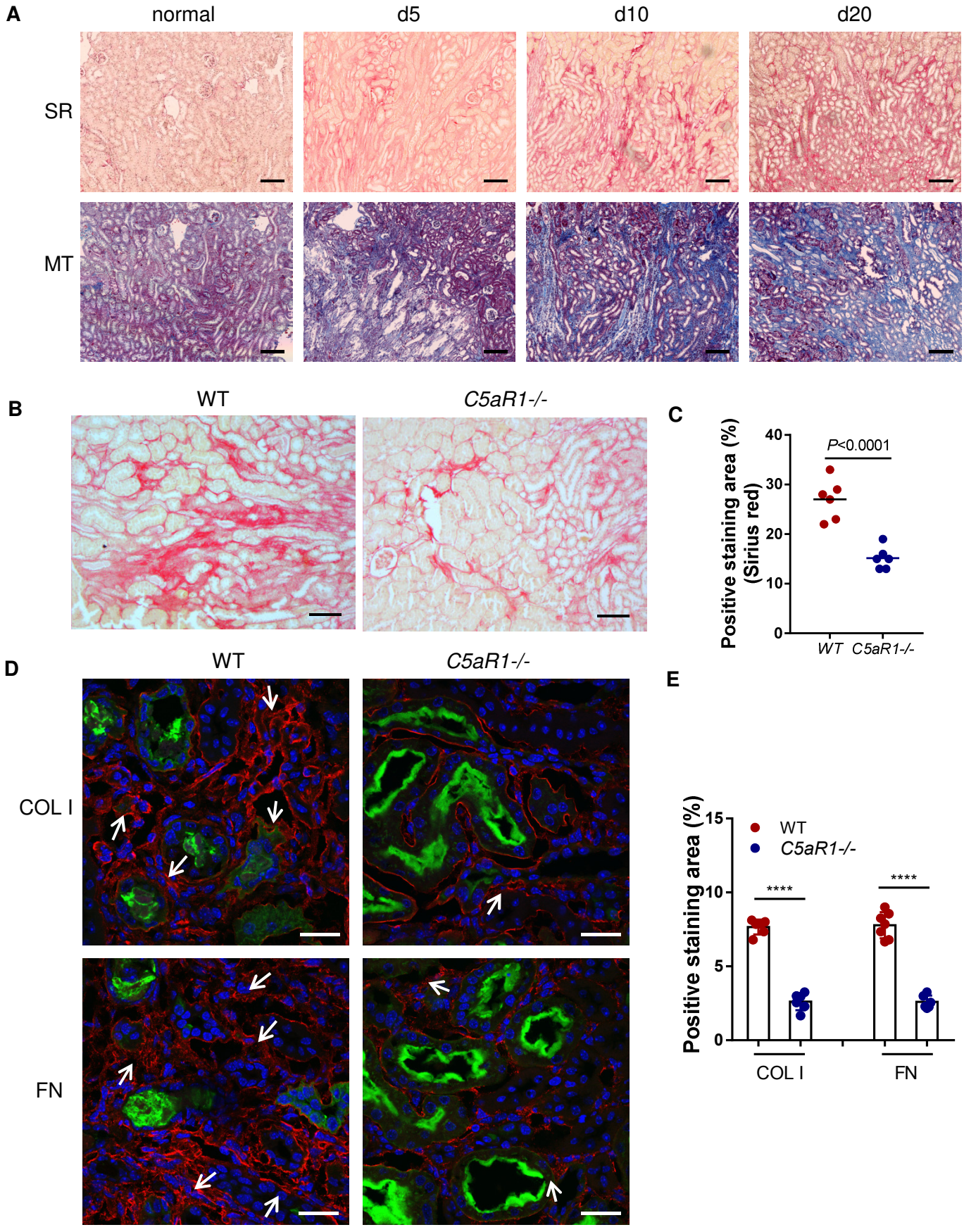
Figure 7. Administration of C5aR antagonist prevents the progression of renal fibrosis in ischemic kidneys.

(A-E) Renal IR injury was induced in WT mice followed by administrating C5aR1 antagonist PMX53 or control (PBS), at d0, i.p. daily. Serum samples and kidneys were collected for the analysis at d10 after reperfusion. (**A**) Representatives of SR staining of injured kidneys with or without administrating the antagonist. Scale bar: 100 μm. (**B**) Quantitative analysis of collagen deposition in the injured kidneys with SR staining. Data are shown as mean ± SD and were analysed by Unpaired two-tailed Student's t test (n=4 mice per group). (**C, D**) Detection of ECM protein (COL I and FN) deposition in the kidneys of mice with PMX53 or control treatment (ctrl) (**C**) Representative fluorescence microscope images of COL I (red), FN (red), and lotus tetragonolobus lectin (LTL) staining in the cortical medullar junction region of kidney sections. Scale bars: 25 μm. (**D**) Quantification of positively stained areas of COL I and FN corresponding to the control and PMX53 treated mice in **C**. Data were analysed by One-way ANOVA with multiple comparisons test (n=6 viewing fields from 3 mice per group, under x600 magnification). (**E**) BUN levels. Data were analysed by Unpaired two-tailed Student's t test (n=4 mice per group). (**F-H**) Additional set of experiments for PMX53 treatment including three groups of mice: control treatment, PMX53 treatment starting at d0 (d0-d9) and PMX53 treatment starting at d3 (d3-d9). Serum samples and kidneys were collected for the analysis at d10 after reperfusion. (**F**) Relative mRNA levels of COL I and FN in the kidneys, determined by RT-qPCR. (G) Quantitative analysis of collagen deposition in the kidneys with SR staining. (**H**) BUN levels. Data in **F-H** were analysed by One-way ANOVA with multiple comparisons test (n=5-6 mice per group). (**B, E-H**) Each dot represents an induvial mouse. The dotted line indicates a normal BUN level. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.

Figure 8. Proposed mechanism by which C5a/C5aR1 interactions promote renal tubulointerstitial fibrosis

Complement activation in response to renal IR insult generates C5a, C5a/C5aR1 interactions may contribute to progression of tubulointerstitial fibrosis through several cellular pathways, these include: i) Recruitment of inflammatory cells and upregulation of the production of pro-inflammatory/fibrogenic mediators by inflammatory cells, leading to a proinflammatory environment, which promotes tubule injury, cellular accumulation, EMT of RTEC and interstitial fibroblast proliferation. ii) Upregulation of the production of pro-inflammatory/fibrogenic mediators by renal RTEC, also contributing leading to a proinflammatory environment. iii) Direct stimulation of fibroblast proliferation and ECM production.

Figure 1. C5aR1 deficiency reduces renal tubulointerstitial fibrosis following renal IR insult



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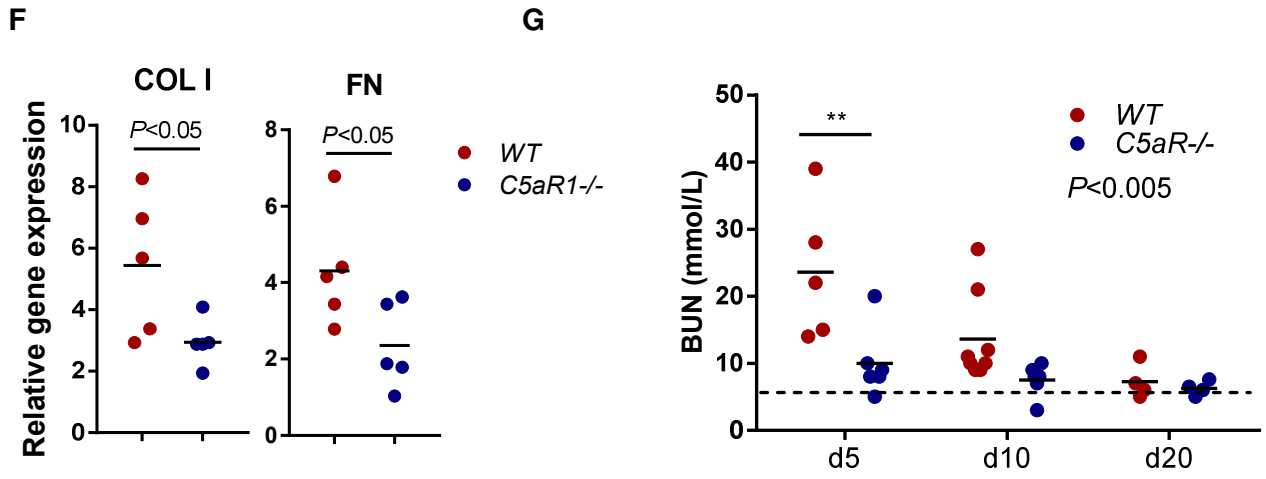
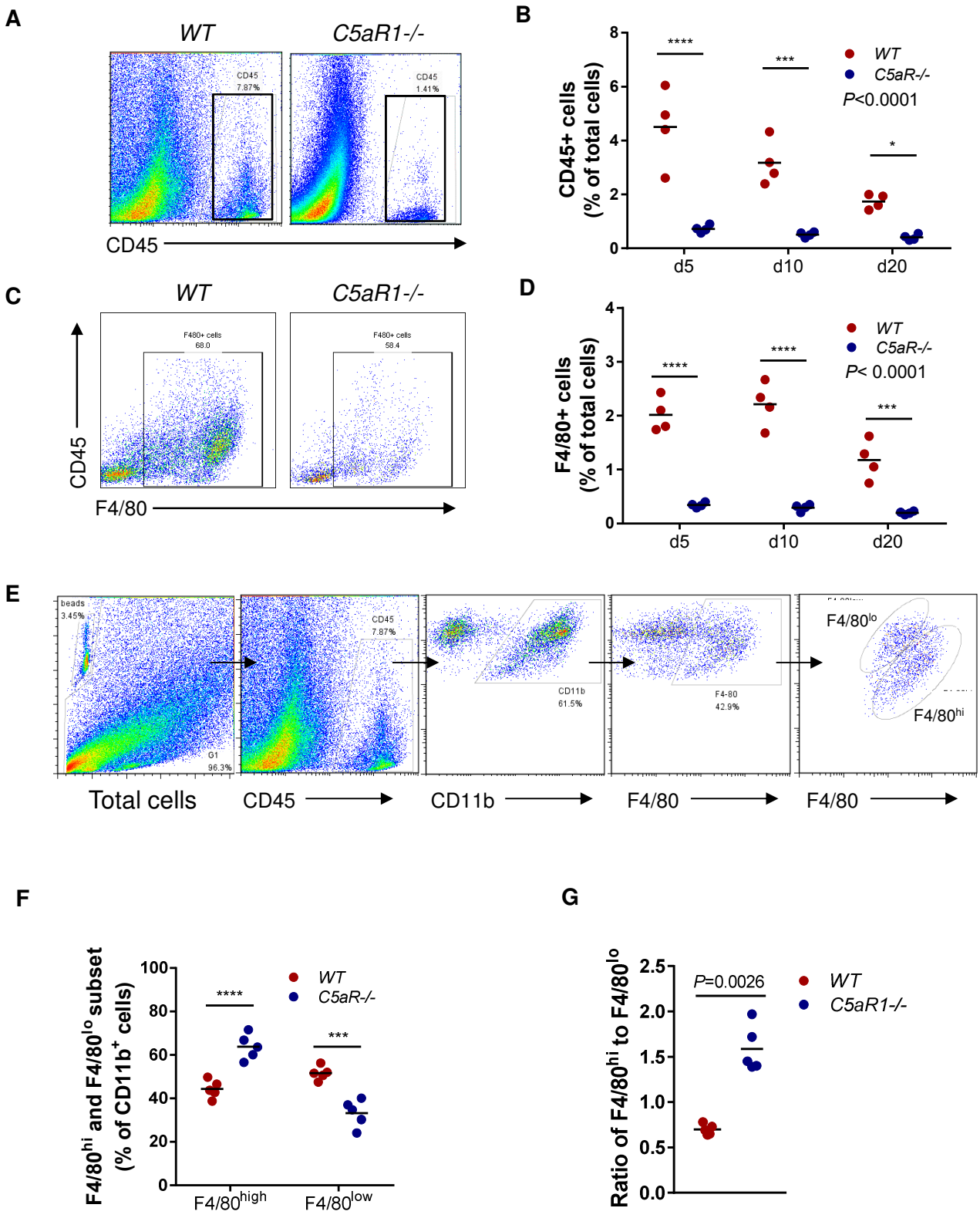


Figure 2. C5aR mediated increased number of infiltrated cells in injured kidneys



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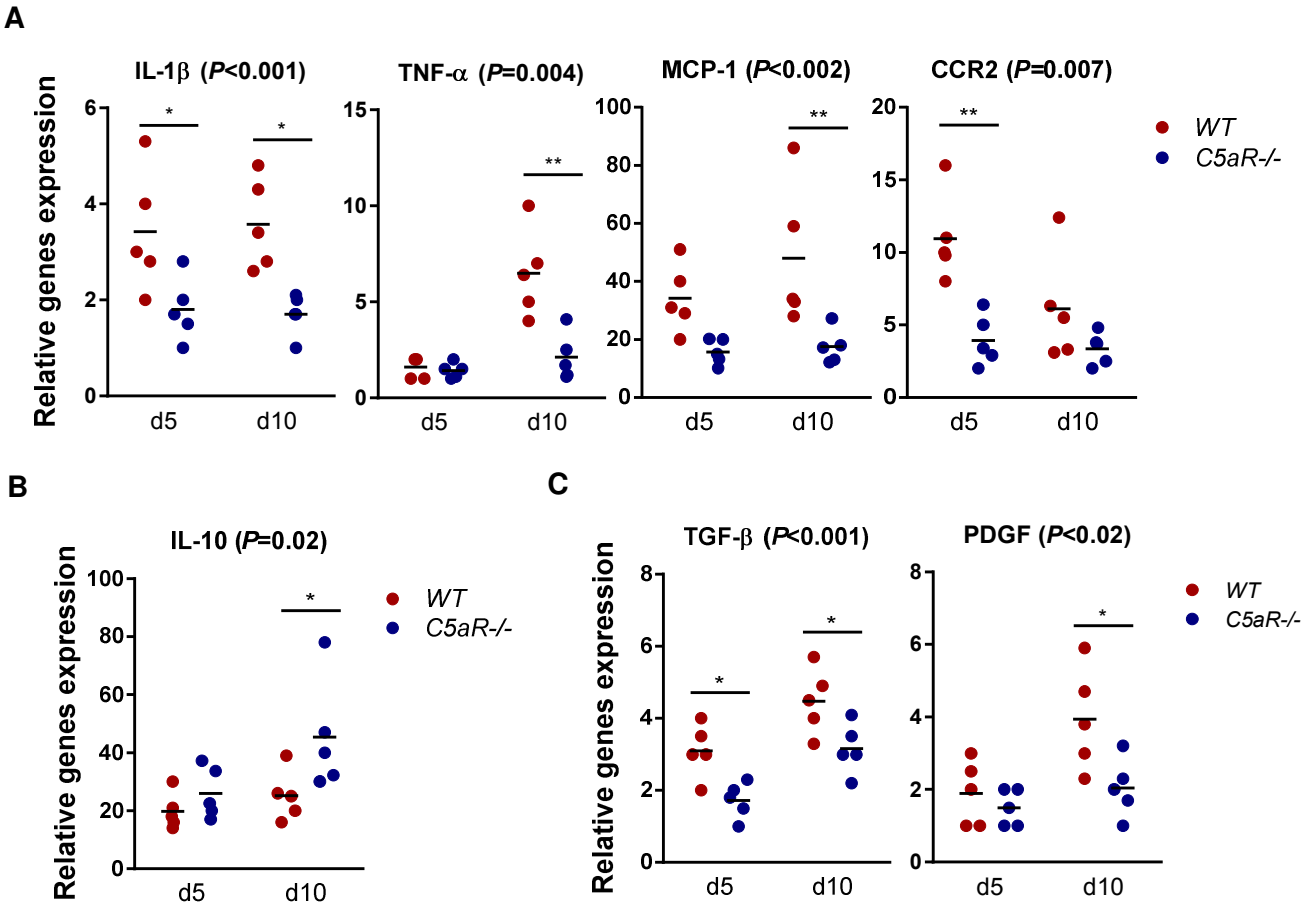

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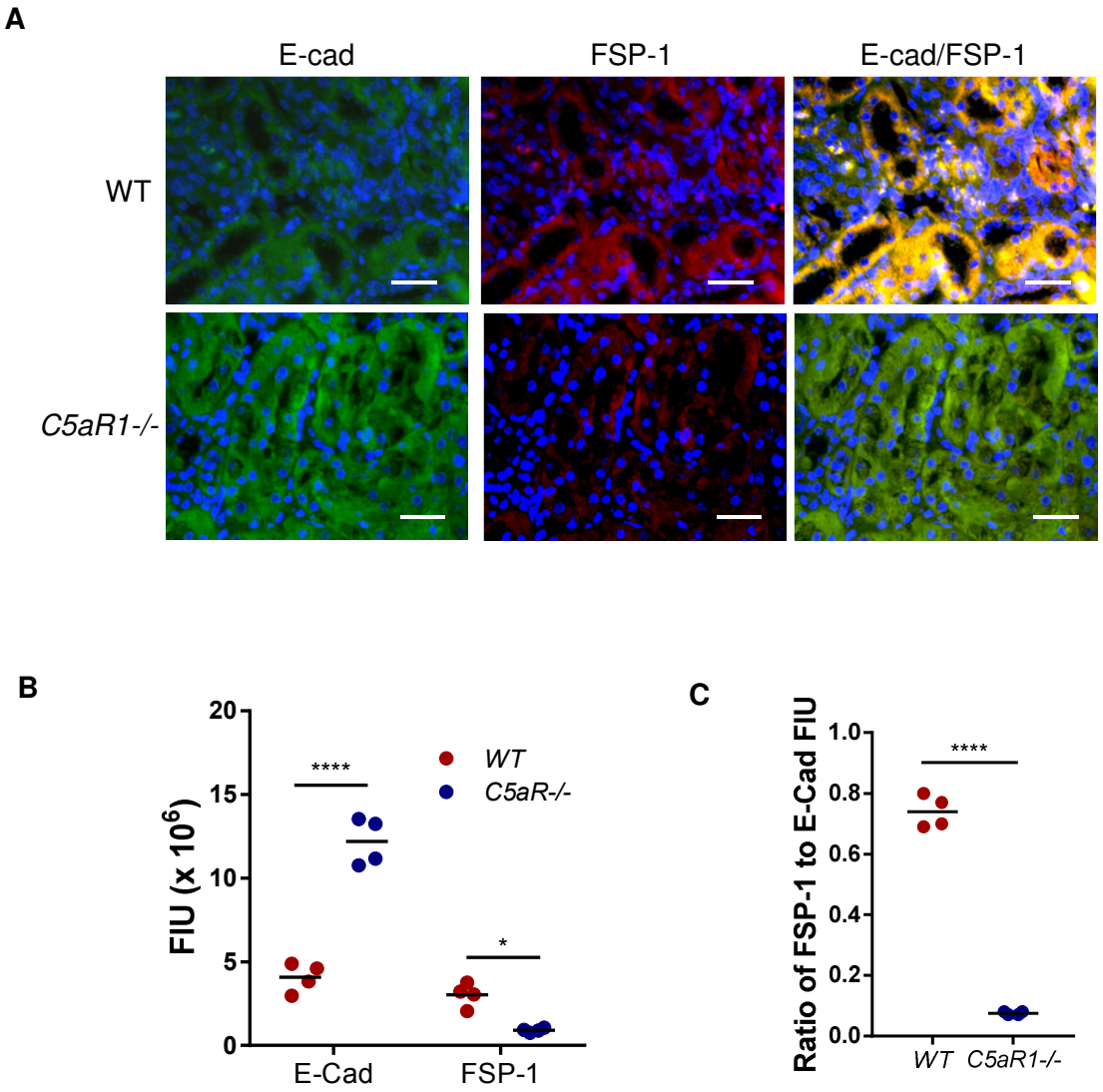

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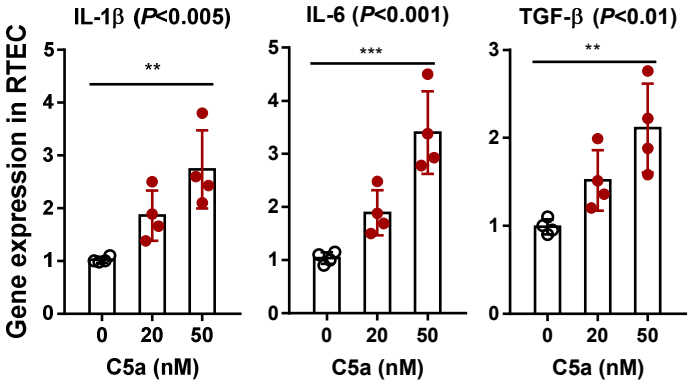
A



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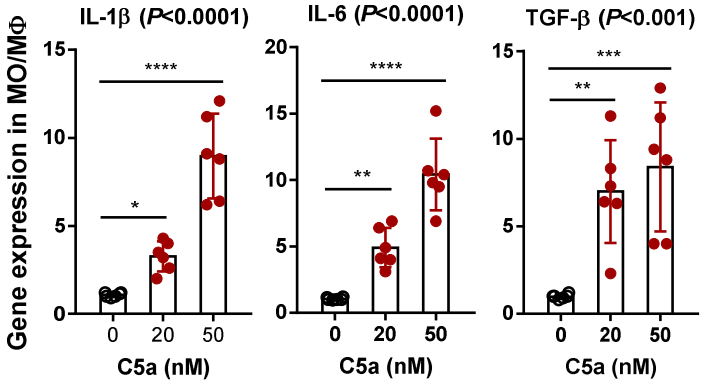

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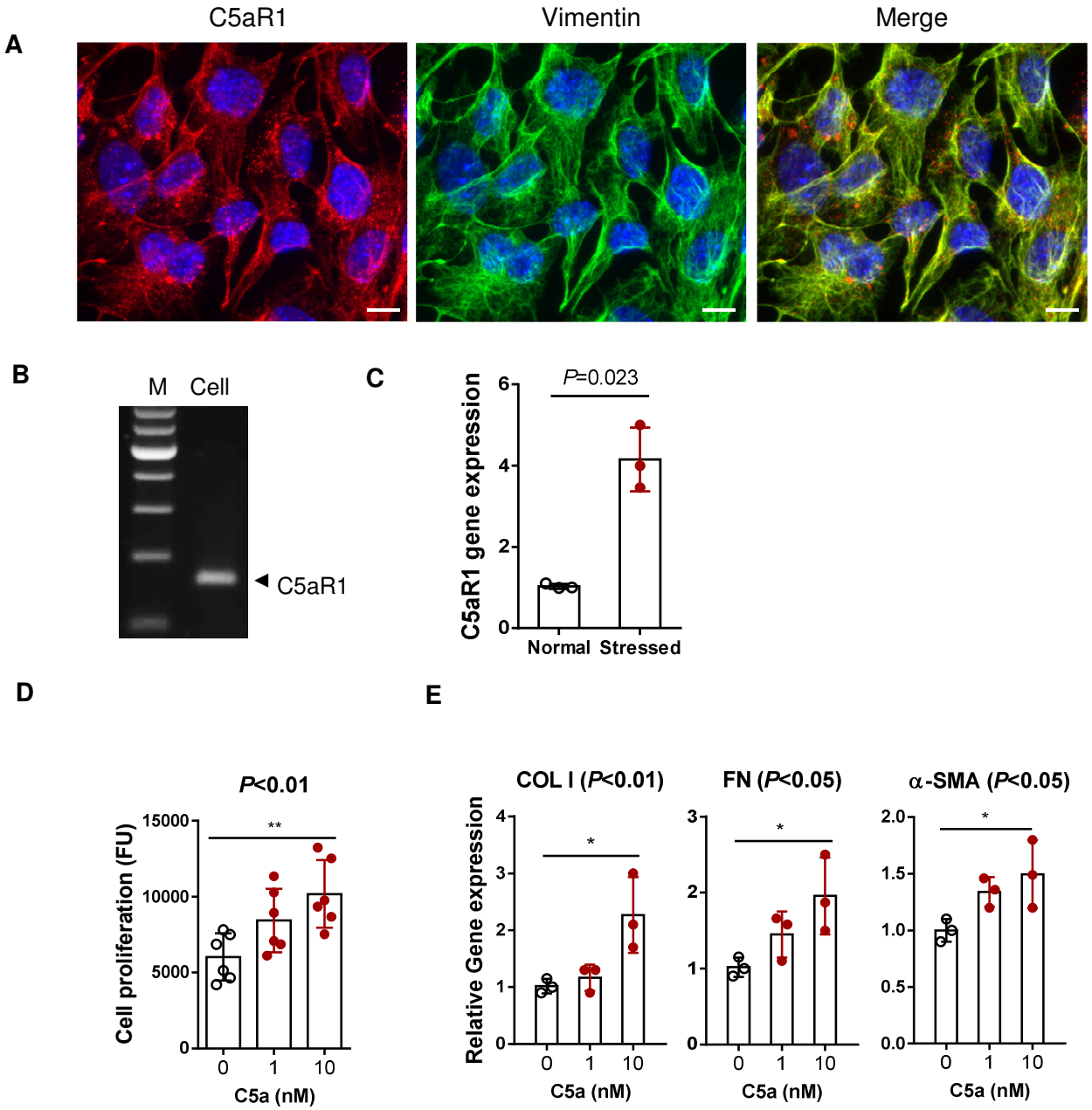

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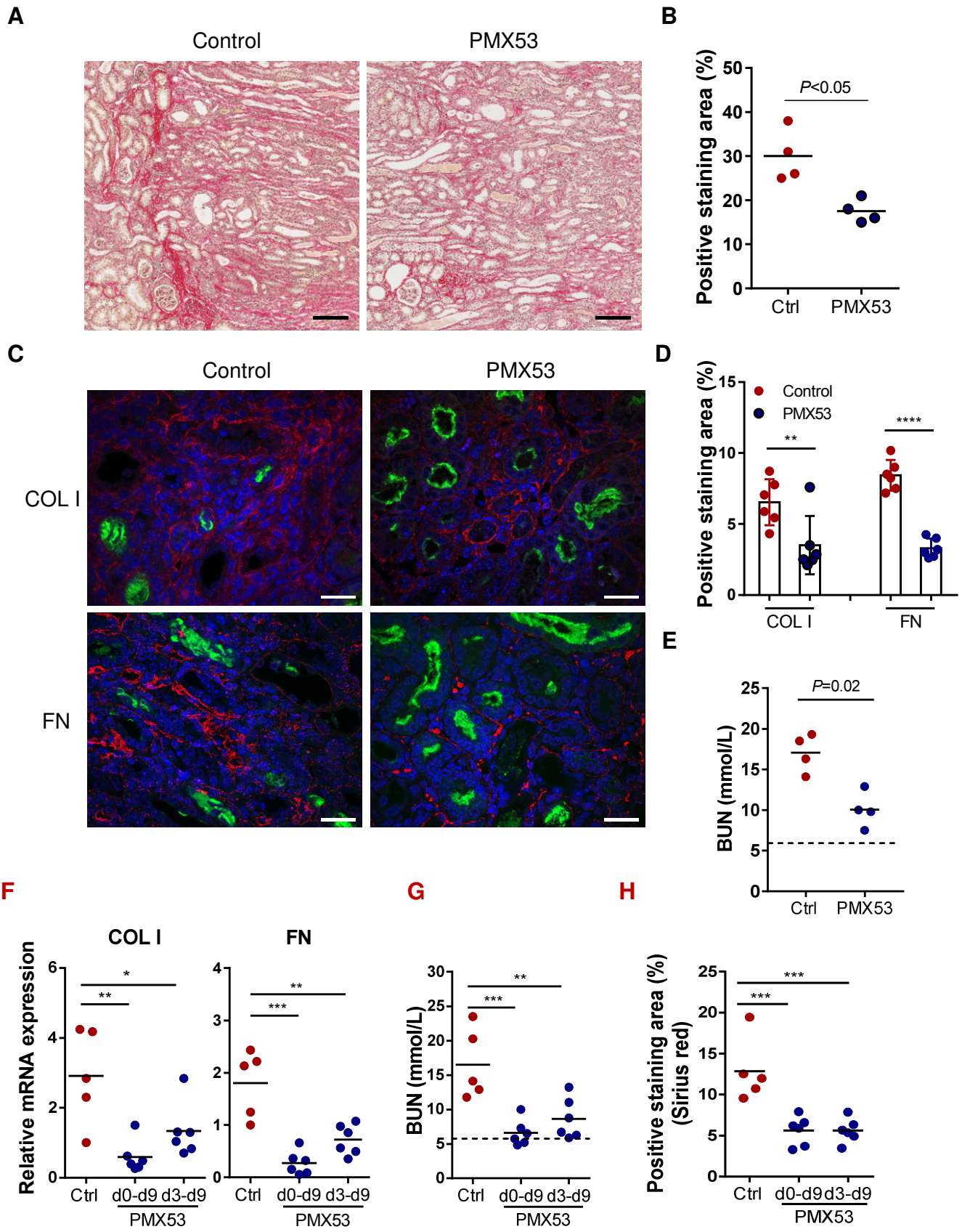

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